The growth of selected mycorrhizal fungi in response to induced water stress[1]

JOHN MEXAL AND C. P. P. REID
Department of Forest and Wood Sciences, Colorado State University, Fort Collins, Colorado 80521

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Three ectomycorrhizal fungi, *Cenococcum graniforme*, *Suillus luteus*, *and Thelephora terrestris* were grown in artificial nutrient media. Water potential of the media was varied by the use of the osmoticum polyethylene glycol (PEG) 4000 and measured by thermocouple psychrometry. *Cenococcum graniforme* was very tolerant of low water potentials and exhibited maximum growth at a potential of −15 bars. Maximum growth of *S. luteus* and *T. terrestris* occurred at −5 bars. The water potential of solutions containing PEG 4000 appears to consist of both an osmotic and matric component, making PEG 4000 ideally suited for simulation of soil moisture stress. It was neither metabolized nor readily absorbed by *C. graniforme* as inorganic salts or sugars might be.

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Trois champignons ectomycorrhizateurs, *Cenococcum graniforme*, *Suillus luteus* et *Thelephora terrestris*, ont été cultivés dans des milieux nutritifs artificiels. Le potentiel hydrique des milieux a été varié à l'aide du polyéthylène glycol (PEG) 4000 et mesuré à l'aide d'un thermocouple psychrométrique. *Cenococcum graniforme* s'est montré très tolérant aux potentiels hydriques faibles et a montré un maximum de croissance à un potentiel de −15 bars. *Suillus luteus* et *T. terrestris* ont eu une croissance maximale à −5 bars. Le potentiel hydrique de solutions contenant PEG 4000 semble résulter à la fois d'une composante osmotique et d'une composante matricielle, ce qui fait du PEG 4000 un matériel idéal pour simuler les tensions hydriques du sol. Cette substance n'a pas été métabolisée ni facilement absorbée par *C. graniforme*, comme des sels inorganiques ou des sucres peuvent l'être. [Traduit par le journal]

Introduction

The full impact that chemical water potential (ψ) may have on the growth of microorganisms has only recently been realized. Microorganisms, like higher plants, differ markedly in their ability to survive soil water stress. Scott's review (26) of food spoilage organisms indicates that some molds grow best at −140 bars and can survive ψ of less than −500 bars. However, these values represent lower extremes and only a select group of microorganisms can survive these potentials. Growth of certain root-rotting fungi (1, 6, 7, 29) is greatest at −10 bars, and does not occur below −80 bars. *Alternaria tenuis* Nees, a saprophyte, also grows best at −10 bars, but can survive ψ of −100 bars. Other fungi, such as certain *Boletus* spp. (28) and *Fomes fomentarius* L. Kickx. (31), are severely inhibited by ψ below −15 bars. Williams *et al.* (35) noted that microorganisms in the soil thrived at about −0.2 bars, but certain groups could still grow at ψ approaching −400 bars. However, most soil fungi studied by Kouyeas (16) had minimum thresholds of −100 bars for growth.

Growth of fungi over a range of water potentials is often normal in distribution (i.e. growth is inhibited by both very high and very low ψ). Growth at high potentials may be inhibited by the lysing of cells caused by uncontrolled water influx (3) or by a limitation of free oxygen (27). Growth at low ψ is inhibited primarily by a loss of cell turgidity (23).

The purpose of this paper is to report the effect increasing water stress has on the growth in pure culture of three ectomycorrhizal fungi: *Cenococcum graniforme* (Sow.) Ferd. and Winge, *Suillus luteus* (Fr.) S. F. Gray, and *Thelephora terrestris* (Ehrh.) Fr. It is well established that mycorrhizas are essential to the survival and growth of trees. Under conditions of poor soil fertility, mycorrhizas greatly enhance both growth and mineral absorption (8). Axenic trees exhibit increased survival rates and growth if inoculated with mycorrhiza-forming fungi (20). While the function of mycorrhizas in mineral nutrition has been somewhat elucidated, the role they may play in the drought resistance of trees has been little investigated. Recently, endomycorrhizas of soybeans have been found to decrease the resistance of water flow into the plant and to increase shoot growth (24, 25). The decrease in

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water resistance was accomplished in part by the increased nutrient status associated with mycorrhizal infections.

It is not well understood what role water plays in the survival and formation of ectomycorrhizas. The fungal population of a *Pinus nigra* forest floor has been studied (27), but no mycorrhizal species were included. However, the species which were identified grew best, on the whole, at about −0.2 bars and were markedly inhibited below −6.2 bars. Generally, it is known that both drought and flooding are limiting factors in ectomycorrhizal formation (28, 36). However, species variability and exact environmental conditions for growth inhibition are unknown.

With present techniques it is not possible to maintain a soil sample at constant ψ. Therefore, to study an organism's response to induced moisture stress, artificial media must be used. The choice of suitable osmotica for the media is of particular importance when one is working with microorganisms. Salts not only upset the water balance of the hyphae, but at high concentrations (low ψ) may introduce a factor of toxicity into the results. At low salt concentrations fungi exhibit improved growth because the solutions are osmotically adjusted rather than matrically adjusted (1, 4). This increase in growth is, in part, due to enrichment on the part of the salts used to adjust the ψ. Sugars commonly used as osmotica (e.g. mannitol) are also easily absorbed and metabolized by fungi (18). Polyethylene glycol (PEG) minimizes these factors (17). However, low molecular weight PEG (e.g. PEG 400) can be absorbed by higher plants and prove toxic. Very high molecular weight PEG (e.g. PEG 20 000) can block passage of water into the plant and thus decrease the ψ of the plant disproportionately. In this study, PEG 4000 was chosen because it is neither readily absorbed nor does it effectively block passage of water into plants (17).

Materials and Methods

Growth of Fungi at Varying Media Water Potentials
Experiment I

Isolates of *C. graniforme* and *T. terrestris* were grown in modified Melin-Norkrans liquid medium (19), hereafter referred to as MMN. Single-strength MMN con-

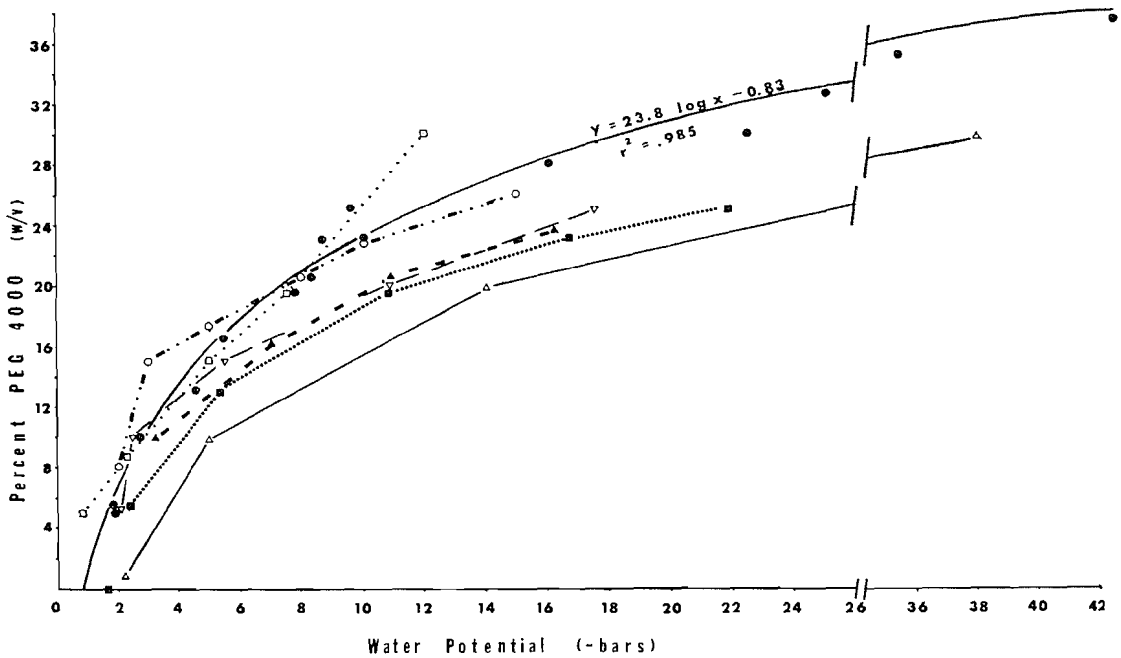

FIG. 1. Water potential measurements of various PEG concentrations by thermocouple psychrometry and freezing-point depression: ● Richards-Ogata psychrometer from unpublished data of J. T. Fisher and C. P. P. Reid: ■ Spanner-type psychrometric measurements of this paper; ▲ freezing-point depression from unpublished data of G. Leavesley and C. P. P. Reid; △ freezing-point depression (32); ▽ freezing-point depression from unpublished data of Bryan Janes (see also 11); □ thermocouple psychrometry (17); and ○ pressure membrane (34).

sisted of $CaCl_2$ (0.05 g), NaCl (0.025 g), KH_2PO_4 (0.50 g), $(NH_4)_2HPO_4$ (0.25 g), $MgSO_4 \cdot 7H_2O$ (0.15 g), Sequestrene 330 (0.0048 g Fe), thiamine HCl (10 μg), glucose (10 g), and malt extract (5 g) per liter of distilled water. Twenty-five milliliters of double-strength MMN were diluted to 50 ml with distilled water and sufficient PEG 4000 to give ψ of 0, −2, −5, −10, −15, and −20 bars. The quantity of PEG required in solution to obtain a specific ψ was calculated from unpublished data of Leavesley and Reid (Fig. 1). As seen from Table 1, calculated potentials did not correspond exactly to measured potentials of the solutions, but for the sake of simplicity they will still be referred to as 0, −2, −5, etc. The medium, autoclaved in 125-ml flasks, was inoculated with 4-mm cores containing hyphae from the perimeter of actively growing colonies on MMN agar. The cultures were incubated on a shaker in a controlled-environment room (23°C and 50% relative humidity). After 13 weeks, the ψ of the hyphae and media was measured with a Spanner-type thermocouple psychrometer (33) and with the Shardakov dye method (15). Determination of ovendry weights (24 h at 100°C) followed the measurements.

Experiment II

Methods were as in experiment I, except with *C. graniforme* and *S. luteus* as inoculants. Growth as ovendry weight was measured after 10 weeks.

Absorption and Metabolism of PEG 4000 by C. graniforme

Experiment III

To determine if PEG 4000 is a suitable carbon source in stimulating growth, *C. graniforme* was grown on MMN agar (1.5% w/v) with different carbon sources substituted for glucose. The treatments included glucose, mannitol, PEG 400, PEG 1500, PEG 4000, and a control containing

TABLE 1

Water potentials (ψ) of MMN culture solutions adjusted with PEG 4000 as determined by Spanner-type thermocouple psychrometer (TP) and the Shardakov dye method (SH)

% PEG 4000 in solution, w/v	Calculated* ψ, −bars	Measured ψ, −bars		
		Initial	Final†	
		TP	TP	SH
0	0	1.6	2.7	1.1
5.5	2	2.5	3.7	3.4
13.0	5	5.3	5.7	6.0
19.5	10	10.8	12.1	10.0
23.0	15	16.7	15.9	11.0
25.0	20	21.8	28.4	>20.0

*Based on unpublished data of G. Leavesley and C. P. P. Reid.
†Measurement of the solutions after 13 weeks of fungi growth.

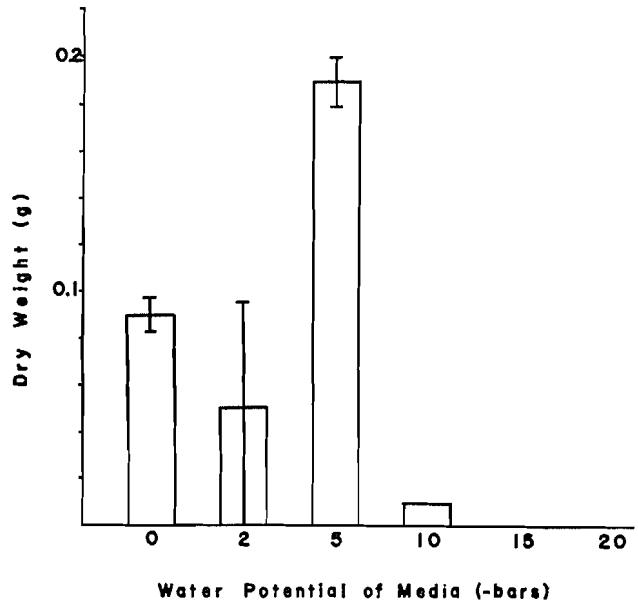

FIG. 2. Growth, ±SE, of *T. terrestris* after 13 weeks expressed on an ovendry-weight basis.

no carbon source save malt extract. The colony diameter was measured periodically, and at the end of 11 weeks dry-weight measurements were made.

Experiment IV

Experiments were conducted to determine if PEG 4000 is absorbed and translocated by hyphae. Hyphae of *C. graniforme* were allowed to grow in petri plates from an agar compartment containing 0.09 μCi of uniformly labeled ^{14}C-PEG 4000 onto agar without ^{14}C-PEG. Only about 150 mg of PEG 4000 were added to the agar, and this amount would decrease the ψ of the agar by less than .01 bar. After hyphae grew into all compartments, two 10-mm core samples of agar and hyphae were removed from all compartments and extracted with 5 ml hot water. Aliquots of the centrifuged extract were added to 10 ml of Instagel[2] and the radioactivity determined by liquid scintillation spectrometry.

Experiment V

Hyphae of *C. graniforme* were allowed to grow from an agar compartment containing appropriate quantities of PEG 4000 to give -2 and -10 bars ψ, about 0.8 and 2.8 g PEG per compartment, respectively, onto agar compartments without PEG. After the hyphae had grown onto the agar not containing PEG, the agar and hyphae were autoclaved, and dry weight measurements of the hyphae made. The hyphae were then analyzed for PEG 4000 turbidimetrically (10). In this technique, the hyphae were homogenized in 8 ml water, and diluted to 10 ml. Proteins were then precipitated with 1 ml $BaCl_2$ (10% w/v), 2 ml $Ba(OH)_2$ (0.3 N), and 2 ml $ZnSO_4$ (5% w/v) in that order. The slurry was centrifuged and a 2-ml aliquot was diluted to 4 ml, and 4 ml trichloroacetic acid (30% w/v in 5% $BaCl_2$ w/v) was added. After 5 min, the optical density was determined on a Spectronic 20 at 635 mμ. Hyphae grown on MMN agar containing no PEG 4000 were also analyzed to serve as a control.

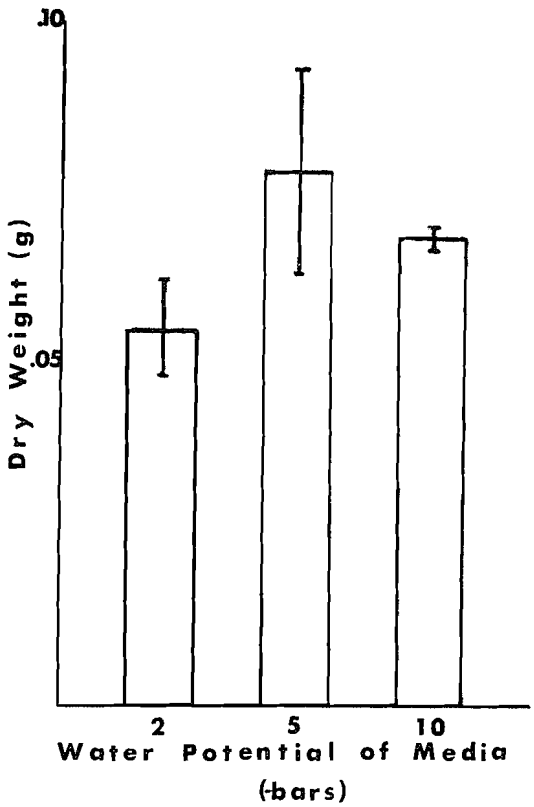

FIG. 3. Growth, ±SE, of *S. luteus* after 10 weeks expressed on an ovendry-weight basis.

[2]A commercial scintillation counting formula of Packard Instruments.

Results

Growth of Fungi at Varying Media Water Potentials

Thelephora terrestris failed to grow at -15 and -20 bars even after 20 weeks (Fig. 2). Maximum growth occurred at -5 bars in the form of pellets. However, as ψ decreased, the pellets became less structured and hyphal strands protruded from the pellets at low ψ. A time lag was associated with initiation of growth at low ψ. There was no lag in cultures above -2 bars; about a 3-week lag in cultures of -5 bars; and about a 6-week lag with cultures of -10 bars.

Suillus luteus was only cultured in solutions at -2, -5, and -10 bars, but growth did not appear to be seriously affected by these ψ (Fig. 3). This mycorrhizal symbiont grew poorly at all ψ, and also failed to show the time lag characteristic of *T. terrestris*.

In both experiments I and II, *C. graniforme* grew in two distinct forms. At high water potentials, the primary growth form was pellets or a hyphal slurry in the solution. With increasing stress more of the growth appeared as a dense hyphal mat on the wall of the flask. This may have been due to spatial limitations for growth. Growth on the wall initiated about 2 cm above the solution. At this height the mycelium was still bathed in the solution as a result of the action of the shaker. Maximum growth in the solution occurred at -10 bars (Fig. 4). Maximum growth on the walls occurred at -15 bars, and total maximum growth also occurred at -15 bars. Maximum growth in the second experiment (Fig. 5) occurred at -10 bars. However, this experiment was terminated early because only general trends were important. Results were similar in both cases.

As with *T. terrestris*, there was also a time lag

associated with the initiation of growth of *C. graniforme*. Growth started immediately in the cultures at 0, −2, and −5 bars. There was a 3- to 4-week lag at −10 bars, and a 7- to 8-week lag at −15 bars. Growth was not evident in the cultures at −20 bars until the experiment was about to be terminated, and then growth did not start in the solution as with the other treatments, but rather on the wall of the flask. Only after a colony was established on the wall did any growth appear in the solution.

Plotting the growth of *C. graniforme* in experiment I on a per diem basis (Fig. 6) accentuates the fact that this mycorrhizal symbiont grows well at low ψ. The dry weight increase per day was much greater at −15 bars than at higher potentials. These values were determined by dividing the total dry weight at termination by the number of growing days beginning with the approximate day growth started (recall the time lags).

Water potential of hyphal samples of all three species as measured with the Spanner-type thermocouple psychrometer, was in close agreement with the final ψ of the media in which they were grown. Water potential measurements of hyphae completely free of interfering medium were not possible. However, large pellets of *C. graniforme* (about 10 mm in diameter) could be sliced and interior tissue sampled. These spheres might afford a partially closed environment for growth, but there were no significant differences in water potential.

Absorption and Metabolism of PEG 4000 by C. graniforme

Colony diameter of *C. graniforme* (expt. III) varied little with different carbon sources in the substrate (Fig. 7B). Final dry weights, however, indicate polyethylene glycols are unsuitable carbon sources (Fig. 7A). Apparently growth occurred only superficially on the agar surface of plates containing no hexose substrate whereas the hyphae thoroughly penetrated the agar if a suitable sugar was available for metabolism.

The movement of ^{14}C-labeled PEG 4000 (expt. IV) occurred readily in the hyphae of *C. graniforme* (Table 2). However, there was considerable variation in the amount absorbed. Similar results have been reported using pepper plants in PEG 4000 solutions (11). The amount of radioactivity in the hyphal (+ agar) samples, however, is less than 5% of the amount present in similar samples from the labeled compartments. Agar cores removed from six compartments initially labeled with ^{14}C-PEG averaged 2062 cpm per core.

Results from experiment V (Table 3) also indicated there was movement of PEG 4000 in the hyphae. Again the amount moved was quite variable and perhaps dependent upon ψ. Whether movement occurred symplastically or apoplastically in the hyphae cannot be concluded from these data. It should be noted that when agar is adjusted to −10 bars with PEG 4000, it requires several weeks to gel and during this time care must be taken in handling plates to avoid con-

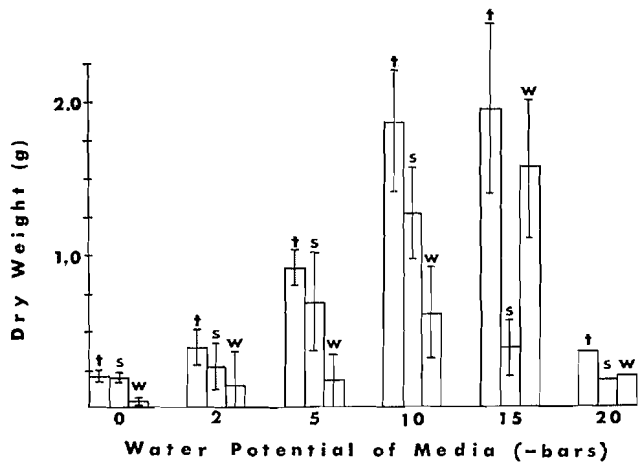

FIG. 4. Growth, ±SE, of *C. graniforme* after 13 weeks expressed on an ovendry-weight basis. Total dry weight (*t*); dry weight in solution (*s*); and dry weight on the wall of the flask (*w*).

tamination of other compartments by PEG.

As evident in Table 2, the extraction procedure was not very efficient even though PEG 4000 is water soluble. Most of the labeled PEG 4000 remained in the precipitate after centrifugation.

Discussion and Conclusions

Mycorrhizal fungi may be more sensitive to environmental stresses, such as drought, than other fungal species reported (4, 6). While some species may grow or even thrive at ψ approaching -40 bars, the species studied here were severely limited by ψ below -15 bars. *Suillus luteus* and *T. terrestris* are intolerant to artificially induced water stress and grow best at ψ near -5 bars. However, *Cenococcum graniforme* grows best at -15 bars. While the results of experiment III indicate maximum growth at -10 bars, it must be noted that the cultures at -15 bars were only growing for about 3 weeks as a result of the time lags. If this experiment had been continued, maximum growth would probably have occurred at -15 bars. These results with *C. graniforme* are supportive of previous work with this symbiont both in pure culture (21) and in association with pine seedlings (9, 37). Other workers (36) have found that *C. graniforme* will succeed other fungi as mycorrhizal symbionts during times of low soil water availability.

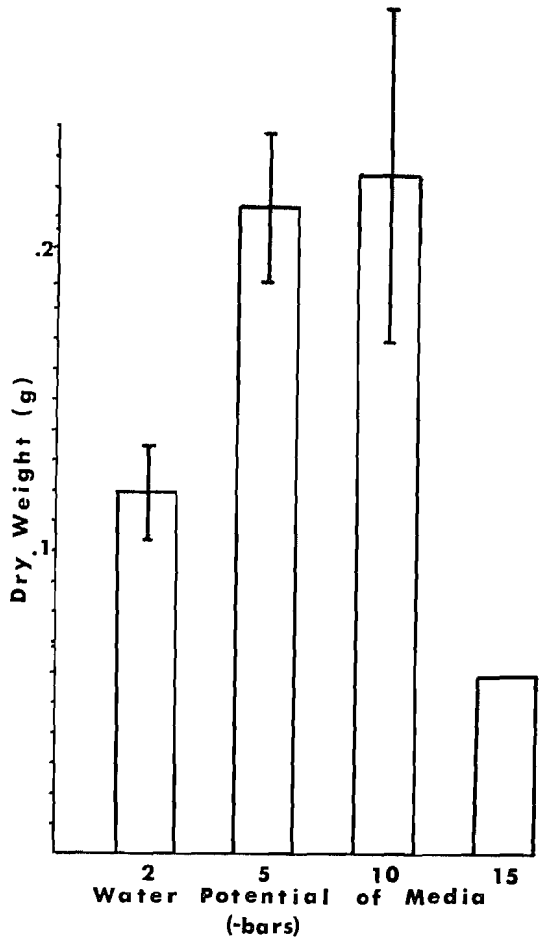

FIG. 5. Growth, ±SE, of C. *graniforme* after 10 weeks expressed on an ovendry-weight basis.

The growth of *C. graniforme* at -20 bars on the walls of the flask before growth initiation in the medium is a matter of speculation. The ψ on the wall should be at least as low as the ψ of the solution. However, oxygen availability might be a factor in the growth initiating on the wall of the flask. At 25°C the oxygen content of a 25% PEG solution (-20 bars) at saturation is only 25% of that of distilled water. It is possible that growth could be limited at this level.

Shemakhanova suggested that the tolerance of fungi to lower osmotic potentials has an apparent advantage over root hairs when soil moisture is reduced (28). Since water moves along a chemical potential gradient from the source (soil solution), through the roots, and to the evaporating surfaces of the leaves, the presence of mycorrhizal fungi which can absorb water against strong chemical potentials could be a very important intermediate structure in imparting drought resistance to plants. The mantle of ectomycorrhizas could serve as valuable insulation between the dry soil and succulent root tissue. In support of this, mycorrhizas have been observed on plants endemic to the Mesopotamian desert. Mycorrhizas were found on such plants as date palm (*Phoenix dactylifera* L.) and *Peganum harmala* L. (13). Although no experimental work was attempted, it was suggested that mycorrhizas supply desert plants with moisture during the dry season.

Apparently PEG 4000 is inert in fungal cells. High PEG 4000 concentrations do not adversely affect fungal growth, since optimum growth occurred in 13% PEG (w/v) for *T. terrestris* and *S. luteus*, and 23% for *C. graniforme*. It was initially thought that the luxuriant growth at these concentrations was the result of some nutritional value afforded by PEG 4000. How-

ever, data presented here indicate that PEG 4000 is not a suitable carbon source for mycorrhizal fungi (expt. III). Growth of *C. graniforme* on substrates containing PEG's as the sole carbon source was markedly inhibited, and may have been totally dependent on the malt extract in the medium, since it will support some fungal growth (18).

The long time lags associated with growth of *C. graniforme* and *T. terrestris* at low ψ do not seem to be explained by the absorption of PEG 4000 to adjust the ψ of the cells, since only a few days at most would be required for the induction of permease enzymes to allow the absorption of PEG 4000. No hypothesis can suitably explain such long time lags. Time lags of a few hours have been reported in fungi (22), and attributed to a loss of turgor within the hyphal cell when the hypha was immersed in hypertonic salt solutions. However, this explanation does not appear feasible in this situation since the time lags were weeks in length rather than hours.

Unfortunately, an accurate estimate could not be made of the amount of PEG 4000 absorbed by hyphae of *C. graniforme*. This could be due to poor extraction or settling of PEG during centrifugation. Poor extraction could also explain the great variability in the results of experiments IV and V.

Polyethylene glycol 4000 is a suitable osmoticum for inducing water stress in fungi. Although it can be absorbed, it is not metabolized as sugars, and not toxic in high concentrations as salts might be. The apparent inertness of PEG 4000 makes it more suitable than osmotica presently used to adjust the ψ of fungal cultures. Polyethylene glycol has been used to induce water stress in higher plants, but as far as known

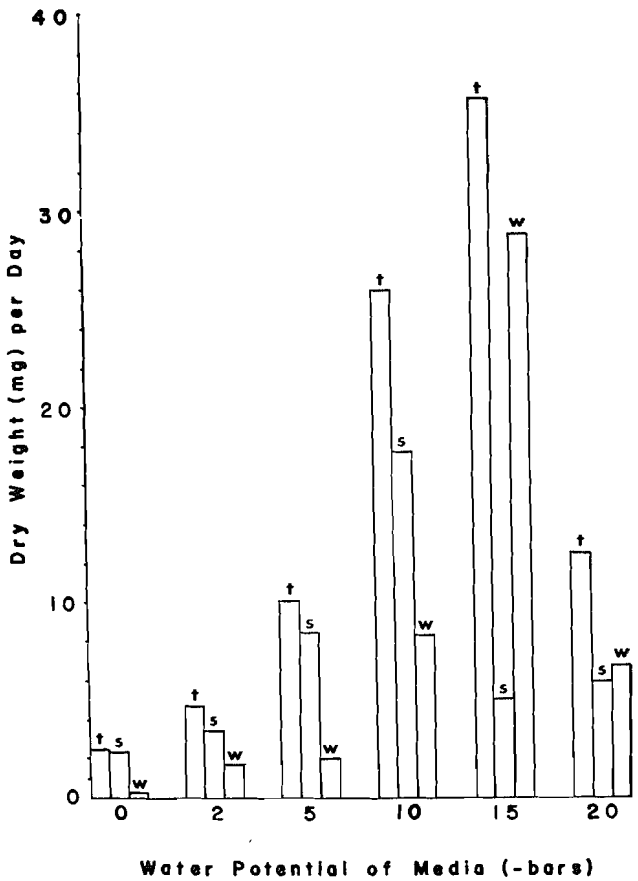

FIG. 6. Growth of *C. graniforme* in experiment I expressed on a per diem basis. Total dry weight was divided by the approximate number of days that the culture showed visible growth characteristics.

it has never been used to test the response of soil microorganisms to water stress. PEG 4000 has been used to simulate drought conditions for seedlings (5, 12, 14, 17), and has been shown to be preferred over salts, mannitol, and lower molecular weight PEG (12, 14, 17). Lawlor indicated that PEG 4000 did not enter plants unless the roots were injured, and impurities present in PEG 4000 did not inhibit growth. Janes (11) found that PEG 400, a low molecular weight

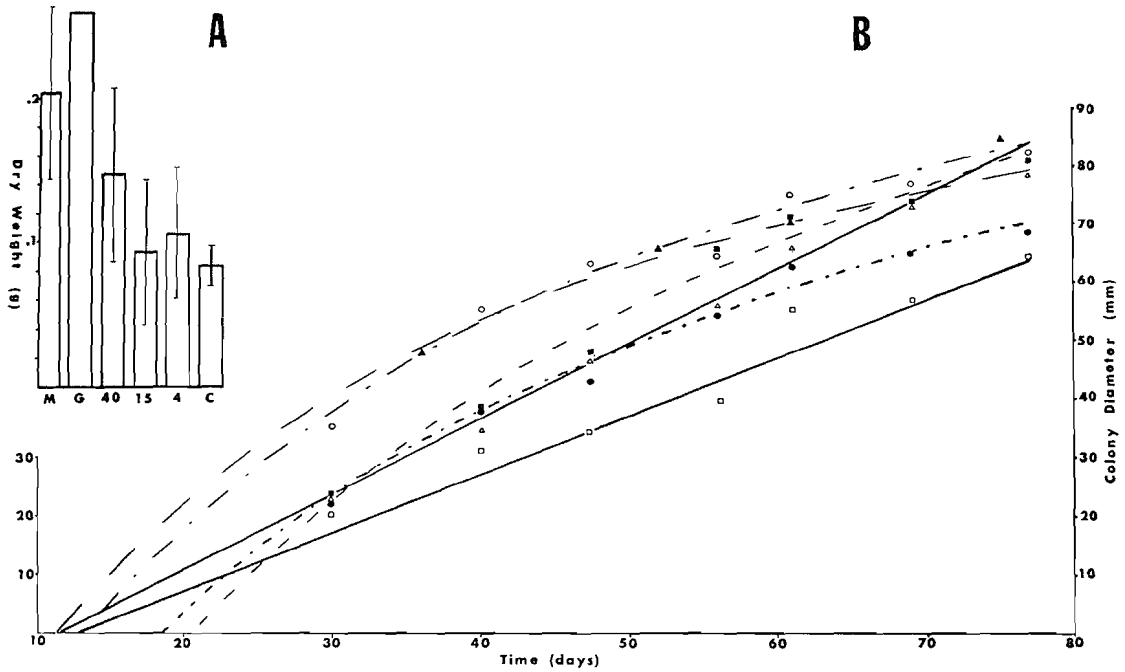

FIG. 7. Effect of carbon source on the growth of *C. graniforme* measured as ovendry weight (A), ±SE, and colony diameter (B). Mannitol (M) △—△; glucose (G) ▲- -▲; PEG 4000 (40) ■- -■; PEG 1500 (15) ○— - ○; PEG 400 (4) ● - · - ●; control (C) □—□.

TABLE 2

Absorption and translocation of ^{14}C-PEG 4000 by hyphae of *Cenococcum graniforme* (expt. IV)

Plate	10-mm core sample	^{14}C activity in extracted core, cpm*		Total PEG 4000 in hyphae, mg/g ovendry wt.†
		Supernatant	Precipitate	
1	a	3.7	15.0	0.16
	b	19.9	344.3	3.16
2	a	7.0	25.6	0.63
3	a	0.0	3.6	0.007
	b	0.0	9.6	0.22
	c	5.0	10.4	0.32
4	a	0.0	43.2	0.33
	b	0.0	112.5	0.85
	c	0.0	40.0	0.30
5	a	0.0	6.0	0.17
6	a	6.3	169.3	1.37
	b	10.6	231.9	1.89

*Counts per minute is net value after correction for background and counting efficiency. Values above 3 cpm are significantly above background at the 1% level.
†Total PEG 4000 per g oven-dry weight of hyphae in each 10-mm core. Based on ^{14}C-PEG 4000 specific activity of 0.6 mCi/g.

homolog, was absorbed by pepper plants only slightly, and the decrease in cellular ψ attributed to the absorption would be only -0.1 bar. It is possible that all absorption of PEG 4000 by higher plants is due strictly to root injury. Polyethylene glycols of molecular weight of 3000 or greater are unable to penetrate cell walls of greenwood cells of Sitka spruce (30). This would seem to indicate that higher plant cell walls including root cell walls are impenetrable barriers to PEG 4000. Unfortunately, the same cannot be said confidently of fungal cell walls.

From an examination of the literature, there appears to be some confusion concerning the actual water potential of solutions containing PEG 4000. Figure 1 takes previously reported ψ values of solutions containing different PEG concentrations and compares them with results obtained in this laboratory. Lawlor (17) found an almost linear function relating ψ to % PEG, while others (32, 34) have found a curvilinear response. Values for PEG 6000 of Williams and Shaykewich (34) are included because Knipe (14) assumed these values could also be applied to PEG 4000 for his germination study. As seen from Fig. 1, the values are in fact applicable. Figure 1 also depicts the results of measuring the ψ of PEG 4000 solutions in this laboratory by freezing-point depression, and thermocouple psychrometry using both the Richards-Ogata (2) and Spanner type. The curve fitted to the Richards-Ogata values is a close approximation of the curve for all values obtained at this laboratory (compare $23.3 \log x - 2$ with $23.8 \log x - 8$). The values obtained using a Spanner-type psychrometer are lower in potential than the Richards-Ogata values. Part of this decrease (about -1.6 bars) is due to the salts present in the nutrient solution which were not present in the PEG 4000 solutions used for the Richards-Ogata determinations. Because experimental error can be reduced to a minimum using the Richards-Ogata psychrometer, we believe these values represent a more accurate determination of the ψ of PEG 4000 solutions.

If this regression curve of the Richards-Ogata data points does represent the actual ψ, then clearly something other than just the osmotic potential is being measured. Gardner *et al.* (6) studied the effect of drying on the ψ of agar gels, and found a logarithmic response similar to our results above. This they attributed to the matric potential component of water potential. This explanation might well be applied to PEG solutions, and if so, PEG would be a good osmoticum for approximating soil moisture stress. High soil moisture stress is due primarily to low matric potential (ψ_m) and not osmotic potential (ψ_s), except in saline soils. Low ψ due to ψ_s may limit water uptake but not water movement. Low ψ_m limits both movement and uptake. Adsorption of water by PEG 4000 would limit uptake in a way different than uptake from salt solutions. Another important consideration is that PEG 4000 is not absorbed to the extent salts are, and thus will not decrease ψ of the cell as much.

TABLE 3

Absorption and translocation of PEG 4000 by hyphae of *C. graniforme* grown on agar at -2 and -10 bars water potential (expt. V)

Water potential of PEG compartment, −bars	Ovendry wt. of hyphae in non-PEG compartment, g	PEG 4000 in hyphae, mg/g ovendry wt.
2	0.180	10.4
	0.068	0.0
	0.065	0.0
	0.143	0.0
	0.071	0.0
	0.170	3.4
10	0.089	143.7
	0.037	90.5
	0.061	47.6
	0.049	8.0
	0.125	42.1

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